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Preparation of Single Cell Detritus from *Laminaria saccharina* as a Hatchery Diet for Bivalve Mollusks

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Abstract A high-yield technique is described for the elaboration of single cell detritus (SCD) from *Laminaria saccharina*, based on the sequential action of C1H, enzymes (endoglucanases and cellulases) and 2 bacteria showing a high degree of cellobiotic, proteolytic, and alginolytic activity (CECT 5255 and CECT 5256). Over 85% of dried particles of *L. saccharina* were transformed into a suspension of free cell and bacterial and detrital particles after 24 hours of bacterial activity with this technique. These particles were less than 20 μm in diameter, constituting a suitable diet for bivalve mollusks. After 72 hours 99% of the total particulate volume consisted of particles less than 20 μm in diameter. Tests of hatchery diets for the seed of clam *Ruditapes decussatus* revealed increases of 54% and 68% for live weight and length, respectively, when SCD from *L. saccharina* was used as the sole dietary component compared with a live phytoplankton diet. However, SCD from *L. saccharina* is not a suitable food for the larvae of *R. decussatus*.

Keywords Biotransformation - single cell detritus - *Laminaria saccharina* - hatchery diet - seaweed - *Ruditapes decussatus*

INTRODUCTION

The implementation of methods for obtaining protoplasts of different macroalgae of the genera *Laminaria*, *Macrocystis*, and *Sargasun* (Polne-Fuller et al., 1986) and *Fucus* (Kloareg and Quatrano, 1987) is a relatively recent development. These techniques generally combine the action of cellulases, pectinases, alginate lyases, and other enzymes extracted from different mollusks (Kloareg and Quatrano, 1987; Kloareg et

al., 1989) and marine bacteria isolated from decomposing fronds of marine macroalgae (Boyen et al., 1990).

Uchida (1996) used the decomposing properties of the marine bacteria *Alteromonas espejiana* to produce protoplasmatic detritus from *Laminaria japonica.* Similarly, Uchida and Numaguchi (1996) applied the same technique to obtain single cell detritus (SCD) from *Ulva pertusa*, *and* showed that these particles are easily ingested by larvae of the clam *Ruditapes philippinarum.* Though no growth studies were performed to test its value as a food, the SCD diameter of 2 to 10 μm, similar to that of various species of phytoplankton such as *Paulova lutheri*, *Isochrysis galbana*, and *Tetraselmis suecica*, allowed these authors to suggest its possible use in mollusk hatchery diets. Similarly, Uchida et al. (1997) used SCD from thalli of *Laminaria japonica* as a diet for the nauplii of *Artemia salina.*

The present paper detscribes our technique for producing SCD from *Laminaria saccharina*, which is on the sequential action of enzymes (endoglucanases and cellulases) and 2 bacteria isolated in our laboratories (strains CECT 5255 and CECT 5256 of the Spanish Type Culture Collection, CECT). We compare performance of this technique with SCD production techniques based solely on bacterial digestion (Uchida and Numaguchi, 1996). In addition, the possible use of this SCD as a diet for the larvae and seed of bivalve mollusks was tested on the clam *Ruditapes decussatus.*

MATERIAL AND METHODS

Preparation of SCD

The SCD was prepared from dried particles (<200 μm) of *L. saccharina* obtained from algae cultivated in suspension and dried in a forced-air oven before being milled and sieved.

Two separate SCD preparation processes were assayed. In the first process, SCD 1 was prepared in 3 steps: an initial stage of hydration and treatment with acid, followed by treatment with enzymes, and finally bacterial digestion. In the second process, enzyme treatment was omitted from the preparation of SCD 2. Bacterial digestion was performed by the strains *Pseudoalteromonas e spejiana* (CECT 5255) and *Vibrio* sp. (CECT 5256) showing a high degree of cellobiotic, proteolytic, and alginolytic activity and isolated in our laboratories from the marine medium. The process of SCD preparation consists of the three stages.

In the first stage, the dried particles of *L. saccharina* were suspended in distilled water as 10% wt/vol, and pH was adjusted to 1.5 by adding HC1 at 17.5%. This treatment took place over a 24-hour period in closed containers on a roller bar agitator at 40°C.

In the second stage, pH was adjusted to 5.4 with 5 N NaOH. For each 10 g of dried particles, 2 ml of the commercial enzyme preparations Viscozime L and Celluclast 1.5 L FG (NOVO NORDISK A/S) was added. The mixture was agitated for 8 hours on a roller bar at 36°C

In the third stage, pH was adjusted to 8 with 5 N NaOH, and the temperature reduced to 27°C. Then 20 ml/L of pure cultures of 10^8 CFU of each of the 2 strains CECT 5255 and CECT 5256 was added. Digestion took place in 5-L bioreactors with a continuous flow of sterile air (5 L/min) and automatic pH control in the range of 7.8 to 8.0, achieved by injecting 1 N NaOH. The bioreactors were maintained at a constant temperature of 27°C and agitated continuously at 100 cycles/min. The rate of bacterial activity was measured by Onset HOBO Event data loggers connected to the automatic pH control equipment.

Samples were taken at the start and finish of the hydration and acid treatment stage, at the end of the enzyme treatment stage (process A), after 24, 48, and 72 hours of bacterial digestion. In process B a further sample was taken after 144 hours of bacterial digestion. The number of particles in these samples was recorded by a Coulter Counter Multisizer with a 500-μm orifice for particles of more than 30 μm in diameter, and a 100-μm orifice for particles in the fraction 2 to 30 μm in diameter.

Feeding Test with Larvae

Straight-hinge larvae of *R. decussatus* with initial average length of 98.66 ± 3.46 μm were used. The experiments were performed in 2-L glass jars, each containing 10,000 larvae in water filtered at 1 μm and sterilized by UV light and the addition of 2.5 mg/L of chloramphenicol. The water was changed and food was added every 2 days. Two types of food were assayed in triplicate: the microalga *Isochrysis galbana* and the SCD 1 from *L. saccarina*, at a density, in both cases, of 6.5 mm^3/L.

Feeding Test with Seed

The experiment was performed over a 3-week period with seed of *R. decussatus* obtained in our laboratories. The seed was kept in 6-L polycarbon vessels, sach containing 3 L of UV-sterilized seawater filtered to 1 μm, the water being changed every day. Food, diluted in 1 L of water per vessel per day, was supplied by means of a multichannel peristaltic pump over a period of 15 minutes every 6 hours. A Coulter Counter Multisizer was used to determine the daily rate of ingestion. Mortality and live weight (LW) of the clams, the latter after clams had been placed on absorbent paper for 5 minutes, were determined weekly. Length was also measured in the initial and final samples.

The food ration was expressed as a proportion of organic weight (OW) of food to clam live weight. Four different diets were assayed in triplicate. Diet A (control), a daily ration of 2% of *I. galbana*, was used as a reference diet. This ratio is considered to produce maximum growth for this species and this type of food (*Pérez Camacho et al., 1998*; *Albentosa et al., 1999*). Diet B was 2% of SCD 1 from *L. saccharina.* Diet C was 4% of SCD 1. Diet D was 6% of SCD 1. Each replicate consisted of 1 g (LW) of clams, with an average length of 5.02 ± 0.86 mm and amean LW of 26.92 ± 0.16 mg.

Statistical Methods

The effect of initial seed size on growth, as determined by a multifactorial analysis of variance, with initial size as a covariable, was not significant ($p > 0.796$). In view of the direct relationship between the mean and the standard variation, LW values were subjected to logarithmic transformation in order to guarantee homogeneity of the variances, and angular transformation was used for those values expressed as a percentage. The differences between diets were analyzed by means of a multiple rank test, using the least significant difference method (*Snedecor and Cochran, 1971*; *Zar, 1974*). The statistics program Statgraphics was used for analysis of variance (ANOVA), multiple rank tests, and comparisons of regression lines between different variables.

RESULTS
Preparation of SCD

Process with Enzyme Treatment

Figure 1 shows the frequency distribution of the size of particles from *L. saccharina* during the different stages in the preparation of SCD with enzyme treatment (SCD 1), expressed as a percentage of total particulate volume. Of the initial dried particles, those with a diameter less than 20 μm accounted for 11.2% of total volume (Table 1), while those less than 10 μm represented 6.1% of total volume. Acid treatment had no appreciable effect on particle size, but after enzyme treatment the proportions of particles with diameters less than 10 μm and 20 μm increased to 24.8% and 32.2%, respectively.

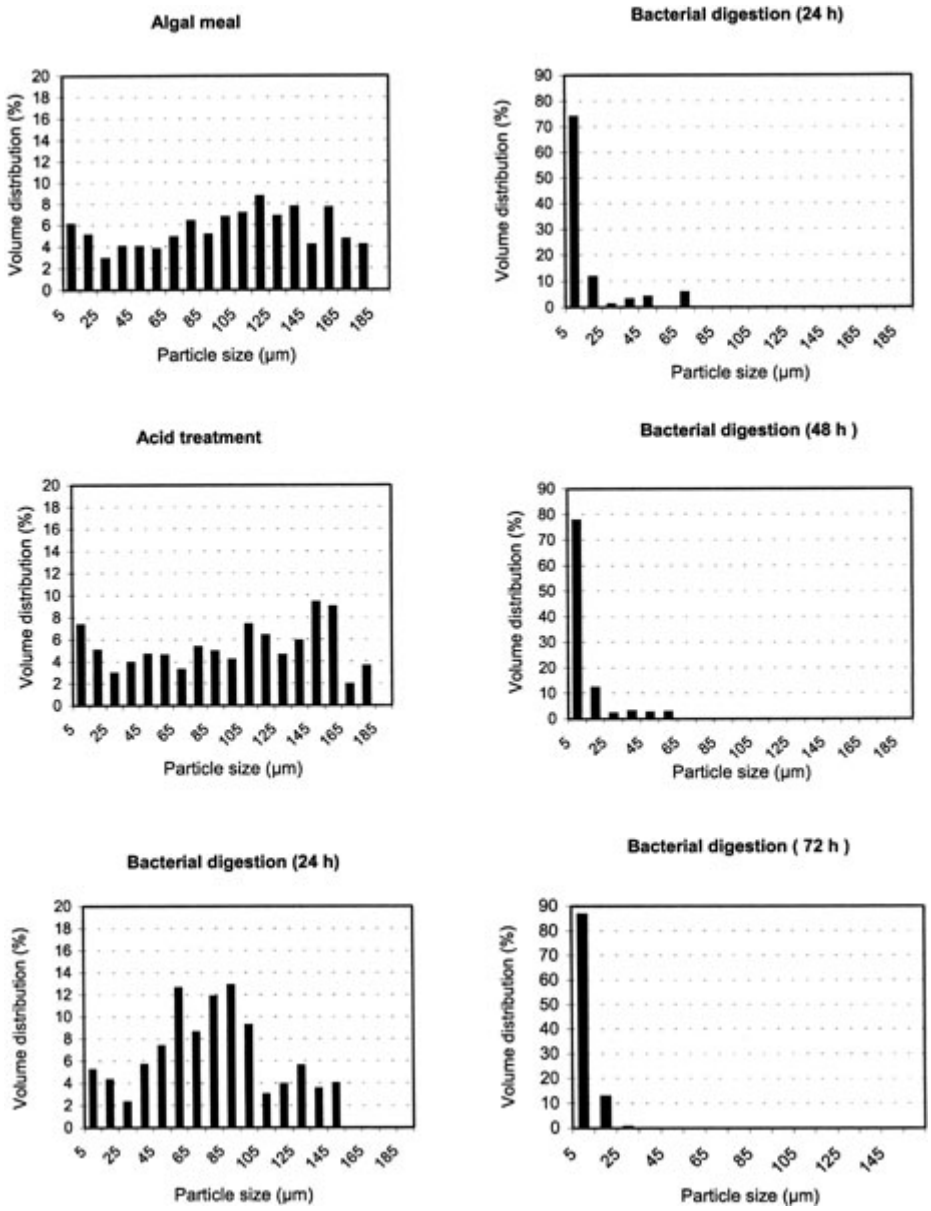

Figure 1 Size distribution of particles of *Laminaria saccharina* (as a percentage of total volume) in various stages in preparation of SCD 1 (with enzyme treatment).

Table 1 Percentage of Total Volume of Particles with Diameters Less Than 10 and 20 μm During Different Stages in Preparation of SCD from *Laminaria saccharina*[a]

Stage of preparation	A		B	
	<10 μm	<20 μm	<10 μm	<20 μm
Dried particles	6.12	11.20	4.88	10.16
Acid treatment	7.36	12.34	4.90	7.96
Enzyme treatment	24.83	32.21	—	—
Bacterial digestion				
24 h	73.99	85.70	5.23	9.54
48 h	77.52	89.77	5.61	12.12
72 h	86.63	99.99	6.80	15.15
144 h	—	—	9.52	20.25

[a]Technique A is with enzyme treatment. Technique B is without enzyme treatment.

Figure 2 shows the number of events for the injection of NaOH into the bioreactors where bacterial digestion occurred. Bacterial activity was concentrated in the first 24 hours of the process. At the end of this period, particles less than 20 μm in diameter represented 85.7% of total volume, and those less than 10 μm in diameter represented 74.0% (Table 1). After 72 hours particles less than 10 μm and 20 μm accounted for 86.6% and 99.9% of total volume, respectively. The end product in this process was an aqueous suspension containing 10% dry weight, with a concentration of 3×10^8 ml^{-1} particles of between 2 and 20 μm in diameter.

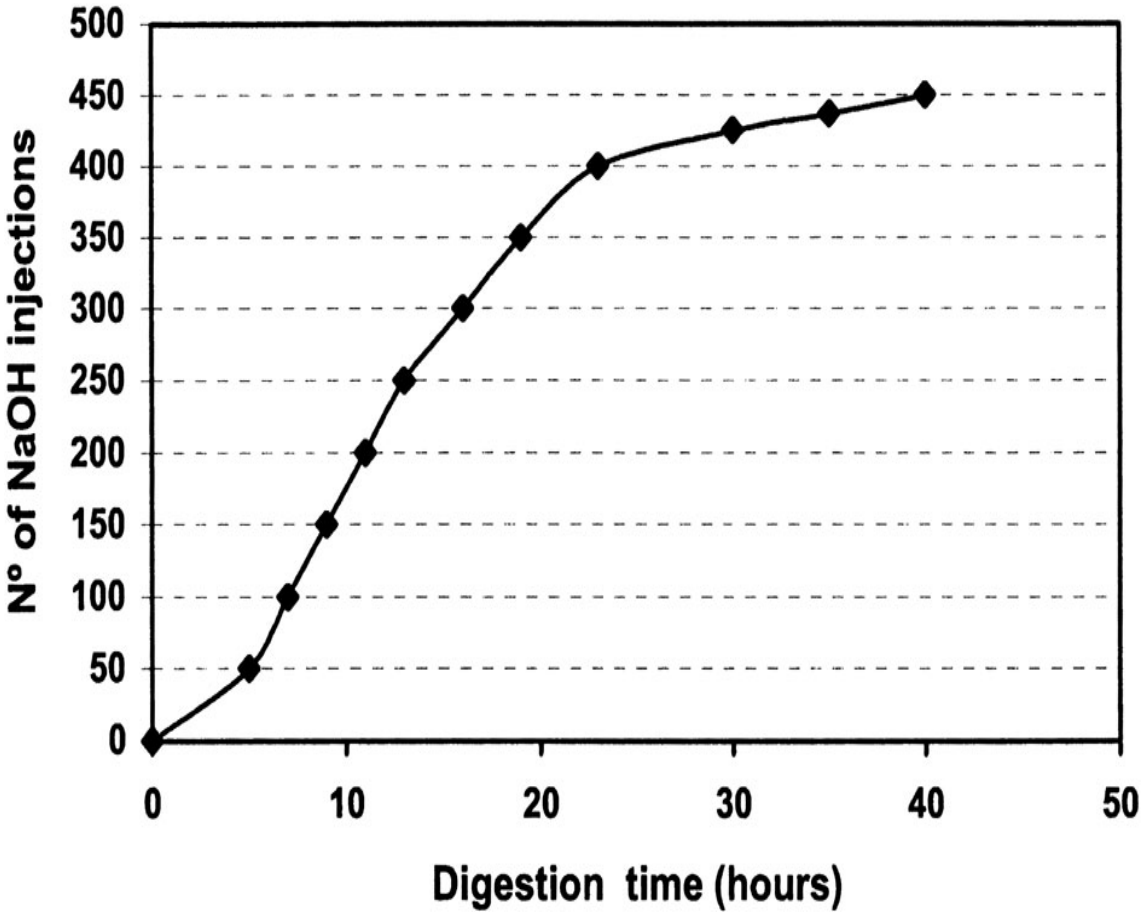

Figure 2 Bacterial activity in preparation of SCD from *Laminaria saccharina* with enzyme treatment: number of events of NaOH injection into the bioreactors in which bacterial digestion takes place.

Microscopic examination of SCD 1 revealed a suspension of free algal cells of a bright green color and the shape of a more or less regular rectangular prism, measuring approximately $11 \times 7 \times 4$ μm. These cells were free of any surrounding layer of cellulose. The suspension also contained bacteria and irregular particles, of a smaller size than the above-mentioned cells, resulting from the decomposition of the cellulose walls (Figure 3).

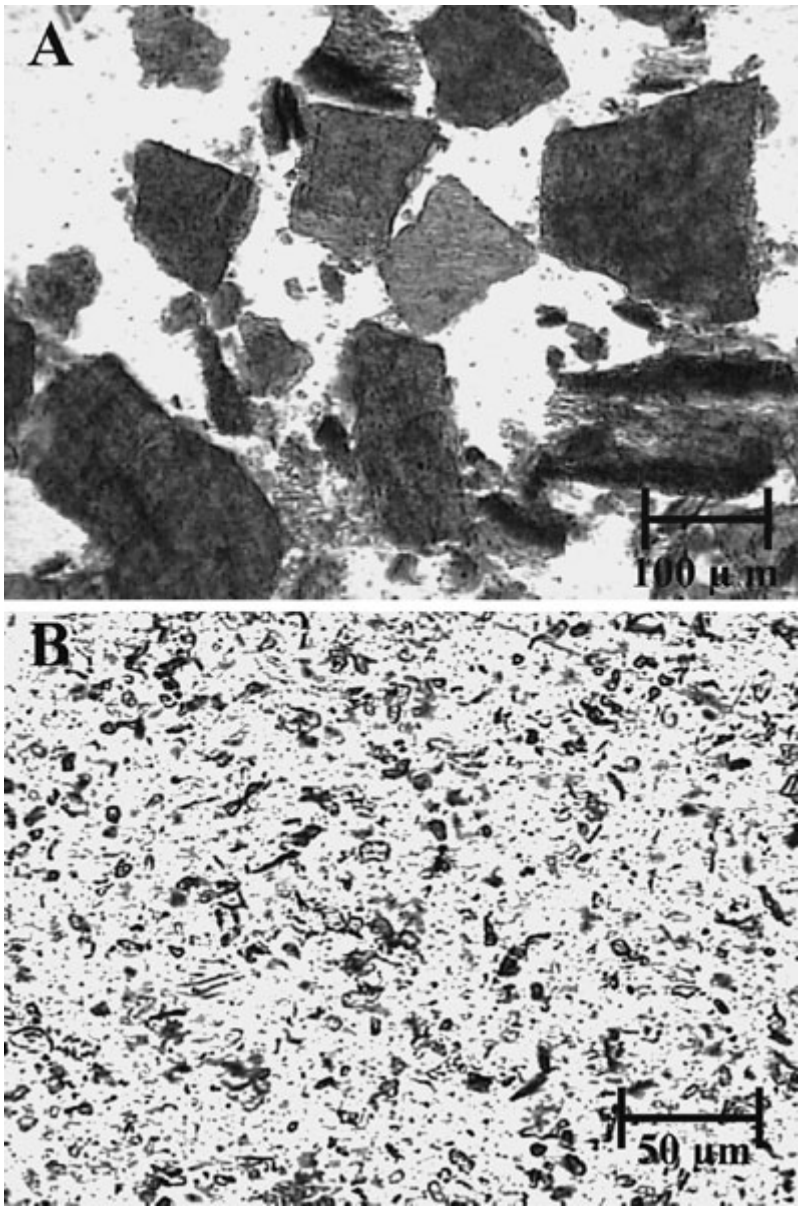

Figure 3 Microscopic aspect of decomposition of particles of *Laminaria saccharina*. **A**: Particles at the start of the process. **B**: Free algal cells, cellular detritus, and bacteria after enzyme treatment and 48 hours of bacterial digestion.

Process Without Enzyme Treatment

Figure 4 shows the frequency distribution of the size of particles from *L. saccharina* during the different stages in the preparation of SCD without enzyme treatment (SCD 2). In this case, of the total volume of initial dried particles, those with a diameter less than 20 μm accounted for 10.2% (Table 1), while those less than 10 μm represented 4.9%. Acid treatment had no appreciable effect on particle size, and the effect of bacterial activity was much less intense than in the process with enzyme treatment. As a result, after 72 hours of bacterial digestion, particles less than 10 μm in diameter represented only 6.8% of total volume, and those with a diameter less than 20 μm,

15.15% (Table 1). After 144 hours these values had increased to 9.5% and 20.25%, respectively.

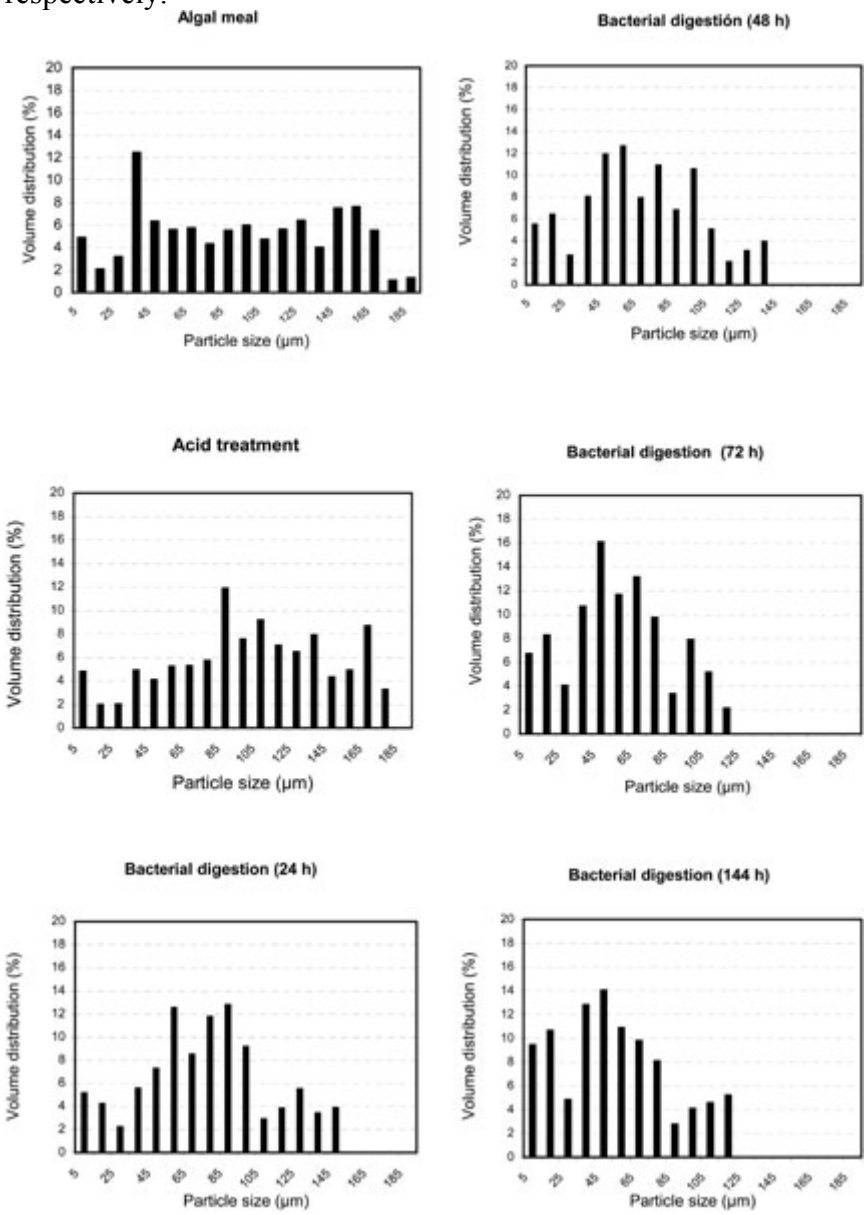

Figure 4 Size distribution of particles of *Laminaria saccharina* (as a percentage of total volume) in various in preparation of SCD 2 (without enzyme treatment).

In addition to the organic detritus and bacteria that appear in SCD 1, microscopic examination of SCD 2 revealed the existence of larger particles consisting of groups of various cells within a polygonal layer of cellulose walls characteristic of the tissues of *L. saccharina.*

Feeding Test with Larvae

The larvae fed on a diet of *I. galbana* developed normally, reaching an average length of 208.9 ± 12.8 μm after an 18-day culture period, with survival rate above 80%. Food ingestion was normal in the larvae fed on SCD 1 from *L. saccharina*, and microscopic examination showed the digestive tubes to be full, but the larvae shell size showed no

variation throughout the experiment, while the volume of visceral mass decreased and the mortality rose to 100% by day 10 of the culture period.

Feeding Test with Seed

Growth of the seed of *R. decussatus*, measured in terms of the increase in LW for mean values of each diet, is shown in Figure 5. The clams fed on live phytoplankton registered higher growth rates than those fed on the different rations of SCD 1 from *L. saccharina.* Accordingly, at the end of the experimental period, the clams fed on daily rations of SCD 1 from *L. saccharina* of 2%, 4%, and 6% registered an increase in LW of 43%, 45%, and 54%, respectively, as a percentage of the increase registered by the clams fed on a diet of live phytoplankton. Comparison of the various diets, after logarithmic transformation to homogenize the standard deviations, shows the differences to be statistically significant (ANOVA, $P < 0.0001$). The multiple rank test shows significant differences ($P < 0.05$) between diet A and all the other diets, as well as between diets B and D, while the differences between diets B and C, and C and D, are not statistically significant (Table 2).

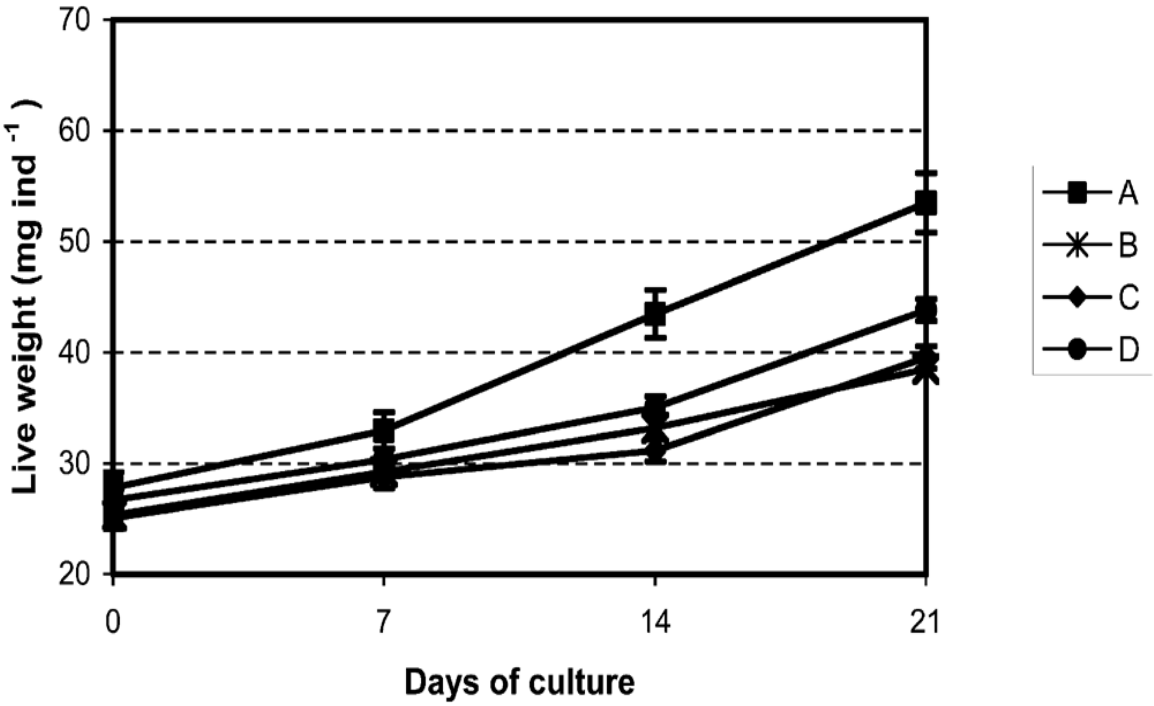

Figure 5 Increase in live weight (LW) of the seed of *Ruditapes decussatus* fed on a daily ration of 2% of *Isochrysis galbana* (**A**), and 2% (**B**), 4% (**C**), and 6% (**D**) of SCD from *Laminaria saccharina* (ash-free dry/live weight). Mean ± standard deviation.

Table 2 Daily Increase in Live Weight and Length of the Seed of *Ruditapes decussatus* Fed Over Three-weeks on Daily Ration of *Isochrysis galbana* of 2%, and of SCD from *Laminaria saccharina* of 2%, 4%, and 6% (ash-free dry weight/live weigh)

Diet	LW increase (mg/d)	Homogeneous groups[a]			Length increase (mm/d)	Homogeneous groups[a]		
I. galbana 2%	1.523 ± 0.154	X			0.086 ± 0.007	X		
SCD 6%	0.803 ± 0.072		X		0.058 ± 0.004		X	
SCD 4%	0.706 ± 0.030		X	X	0.050 ± 0.004		X	X
SCD 2%	0.626 ± 0.042			X	0.044 ± 0.004			X

[a]Xs in the same column indicate no significant difference between the variables (homogeneous groups). Mean ± standard deviation.

The increase in length of the clams fed on rations of SCD from *L. saccharina* of 2%, 4%, and 6% follows a similar pattern, representing, respectively, 51%, 58%, and 68% of the increase registered by the clams fed on the diet of pure phytoplankton (Table 2). In this instance, the multiple rank test shows significant differences ($P < 0.05$) between diet A and all the other diets, and between diets B and D, while the differences between diets B and C and those between diets C and D are not statistically significant (Table 2). The percentage of food ingested was similar in all the diets assayed, ranging between 73.5% ± 8.77% for the phytoplankton diet and 67.6% ± 8.7% for the 2% SCD diet. The differences between the ingestion percentages for the different diets were not statistically significant.

Mortality was less than 10% in all cases, and there were no significant differences between the diets (ANOVA, $P > 0,05$).

Discussion

The present paper compares 2 techniques for preparing SCD from *L. saccharina* treated with acid: one based on the sequential action of enzymes and 2 bacteria, strains CECT 5255 and CECT 5256; the other based on bacterial digestion alone. The first of the 2 techniques was registered by the Spanish Oceanographical Institute in the Spanish Patents Office in May 2002 under the number P200200993.

Initial acid treatment hydrates and spongifies the organic structures of the dried particles of *L. saccharina.* This prepares the particles for subsequent treatments, by transforming the insoluble salts of alginic acid (mainly calcium alginate) into soluble alginic acid, as well as producing a certain degree of general hydrolysis of complex polysaccharides and sterilizing the product.

The function of an enzyme attack is to start breaking down the polysaccharides and cellulose fibers in the cell walls. Bacterium CECT 5255 is *Pseudoalteromonas espejiana*, of a highly alginolytic nature, similar to the bacteria isolated by *Uchida (1996)* from the thalli of *Laminaria japónica.* CECT 5256 is a marine *Vibrio* of a hitherto unknown species with an extraordinary ability to hydrolyze complex polysaccharides. These 2 microorganisms act in synergy to degrade substrates that, until

now, have proved almost impossible to transform, while preserving the phospholipids in the cell membrane and thus leaving the cells in the tissue of *L. saccharina* practically intact. These characteristics allow the dried particles of *L. saccharina* (with 85% of the biomass consisting of fragments of between 100 and 200 μm in length that are not easily ingested by clams) to be transformed over a 24-hour period into SDC. Within the SDC 85% of the biomass is between 2 and 20 μm in length and consists of free cells with no cellulose walls, individual small particles of detritus, agglomerations of detrital particles, and bacteria. The small size of these SCD particles leads to a high degree of efficiency in their retention in the branchia of bivalve mollusks (*Jørgensen, 1990*) and facilitates their ingestion by clams. As our experiment shows, by means of counts carried out with a Coulter Counter Multisizer, SCD 1 ingestion levels are similar to those obtained for *I. galbana.*

The elaboration process that includes enzyme treatment (SCD 1) is much more effective than the process that only employs bacterial digestion to produce SCD 2 (which is similar to the treatment described by *Uchida in 1996*). Particle size in SCD 2 is inappropriate for a bivalve mollusk diet, since after 6 days of bacterial digestion, 80% of total particulate volume corresponds to particles larger than 20 μm.

An advance report of the technique for preparing SCD with enzyme treatment was presented at the VIII Spanish Conference on Aquaculture (*Pérez Camacho et al., 2002*). In June 2002, when this paper was being prepared for publication, *Uchida and Murata (2002)* published a technique for preparing SCD from dried particles of *Undaria pinnatifida* (<74 μm), based on a fermentative method employing cellulase, one type of acid bacterium, and 2 types of yeast. With this technique, and under optimum conditions, SCD was produced at 5.8×10^7 cells ml^{-1} after 6 days of incubation. The technique for producing SCD from *L. saccharina* described in the present paper, which includes the sequential action of acid treatment, enzyme attack with glucanases and cellulases, and bacterial digestion, proved to be more efficient than that of *Uchida and Murata (2002)*, because it takes as its starting point particles of greater size (200 μm) and shortens the production process considerably, given that after 24 hours of bacterial digestion, 85% of the particles in the SCD biomass were between 2 and 20 μm, this proportion increasing to 99% after 72 hours (in *Uchida and Murata 2002*: 63.9%–69.1% of particles between 5.8 and 11.5 μm after 6 days of incubation).

These particles are perfectly suitable for ingestion by larvae and juveniles of *R. decussatus.* The first reference to the possible use of SCD from seaweed in the diet of bivalve mollusks was by *Uchida and Numaguchi (1996)*, although the authors only state that the larvae of the clam *Ruditapes philippinarum* are able to ingest SCD from *Ulva pertusa.* In our experiments, while the larvae of *R. decussatus* were able to ingest SCD from *L. saccharina*, we noted that they appeared unable to digest SCD because shell size did not increase, visceral mass decreased, and the larvae died after several days of cultivation under these experimental conditions, analogous to when larvae are subjected to starvation conditions.

The use of SCD from *L. saccharina* in the diet of juvenile specimens of bivalve mollusks was included in the communication presented by *Pérez Camacho et al. (2002)*. In a later article, *Uchida and Murata (2002)* referred to the possible use of SCD in the diet of juvenile specimens of the Japanese pearl oyster (*Pinctada fucata martensii*). In our experiments, the SCD prepared through the combined action of enzymes and bacteria (SCD 1), when used as the sole component of the diet, proved to be of moderate value as a food for juvenile specimens of *R. decussatus*. Nevertheless, the diet is worth considering because it is a nonphytoplankton food. Increases of 50% in LW and 68% in length were obtained for this SCD, expressed as a proportion of the increase given by a 2% diet of live phytoplankton, which is considered to be the diet that produces maximum increase rates (*Pérez Camacho et al., 1998*; *Albentosa et al., 1999*). These increases are higher than those obtained for other alternative foods such as cornmeal or cornstarch (*Pérez Camacho et al., 1998*), and wheatgerm flour (*Albentosa et al., 1999*), for which increases of 13%, 11%, and 29% in LW were obtained, respectively, expressed as a percentage of the increase corresponding to a 2% diet of live phytoplankton.

Hitherto, all attempts to substitute 100% of the live phytoplankton in the diet of bivalve mollusks have proved unsuccessful, although partial substitution (in most cases 50%) by different types of food, such as cereal flours (*Albentosa et al., 1999*; *Castell and Trider, 1974*; *Pérez Camacho et al., 1998*; *Urban and Langdon, 1984*), microcapsules (*Langdon and Bolton, 1984*; *Chu et al., 1987*), and yeast (*Epifanio, 1979*), has been proved possible. Ongoing studies performed in our laboratory in which phytoplankton is partially replaced with SCD from *L. saccharina* have produced encouraging results.

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